In silico patient

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CHAPTER 5

GENERAL DISCUSSION

In the last decade, systems medicine has grown from a philosophical concept into a thriving new scientific field that studies how physiological functions emerge from the interactions between molecules, cells, and tissues in the body, and puts them in medical research, and practice. This concept of synergy, a whole is more than a sum of its parts, dates back as far as ancient Greece [1,2], and is the origin of the holistic approaches to studies of living organisms. However, initially, holistic approaches to study human physiology remained mostly qualitative and unable to decipher exact mechanistic explanation to the observed phenomena. With the rise of biochemistry at the beginning of 20th century, scientists have revisited Bacon's methodological reductionism [3], according to which breaking the system down and studying its primary components would allow one to understand the system fully as a whole. This approach has brought new methods to study human physiology and diseases, which led to many groundbreaking discoveries such as the structure and the code of the DNA [4,5] and enzyme kinetics [6,7]. However, breaking the living cell into its basic components and studying them in isolation removes the information on interactions between them. Systems biology addresses this problem and tests the assumption that the system can be understood from the interaction of the parts. After all, the information about the building blocks: parameters, variables, and relations, used to construct models in systems biology comes from the reductionistic studies. However, since systems biology looks at the composite characteristics of the problem, rather than its individual components, many argue that systems biology, is in fact, a return to holistic ideals [8–10]. This statement can be disputed since rather than studying the system as an unbreakable whole, systems biology aims to understand the emergence of biological functions from interactions of the components[11]. In this, we can see that the holistic and reductionist approaches, even though they started as opposite concepts, with current technological advancescan be integrated in studies of human diseases [9].

The 21st century has brought a breakthrough in genome sequencing methods that allowed a human genome to be fully sequenced in 2000 [12]. In parallel various 'omics' techniques for comprehensive analysis of transcriptomes [13], proteomes [14], metabolomes [15], or even fluxomes [16,17] were developed and optimised. With the rapid growth in the data acquisition methods, we are now able to gather and store an unprecedented amount of patient-specific data. This data collection aims to monitor the health of an individual better, to diagnose and understand diseases, and to propose the most efficient treatment for each patient. This aim requires robust methods to integrate and interpret the available data. In recent years many supervised, semi-supervised and unsupervised methods of data integration have been developed [18–21].

However, further optimisation and developments are needed to create truly robust tools to work with the heterogeneous omics data. In this thesis, I addressed this problem using metabolic models as tools for multi-level data integration, hypothesis generation, and studies of disease mechanism in a set of inborn errors of metabolism. In this chapter, the results of my studies will be discussed in the context of the systems medicine framework.

"DATA! DATA! DATA! (…) I CAN'T MAKE BRICKS WITHOUT CLAY."[1]

Various methods are under development to approach data integration and interpretation in a systems medicine framework. As reviewed in **Chapter 1**, they are divided into two distinct groups: 1) artificial intelligence (AI)-based knowledge discovery and 2) simulation-based analysis using mechanistic models. The first group has shown great promise for assisting an image-based diagnosis of patients [22–24]. However, its "black-box" nature limits its usability in problems that require a mechanistic understanding of the system. To address such problems, the second group of methods is commonly used [25–31]. Both groups share one "weakness" – data dependency. AI-based algorithms usually require big datasets to find the specific patterns emerging (unsupervised), or training sets that allow the algorithm to learn what pattern to look for (supervised), which makes them ill-suited for rare diseases or patient-specific questions. Similarly, mechanistic models require a priori knowledge, or at least a hypothesis, about the network topology, its components, and their interactions. As illustrated in chapters 2 and 3 of this thesis, auxiliary pathways or transport systems are often still enigmatic and may be misrepresented in the metabolic network. Furthermore, models should be validated using robust datasets before they can be personalised for patient-specific studies, as seen in chapters 2-4. The amount and the type of data required for parametrisation and validation of the models depend on the type of the models used.

In **Chapter 1**, I introduced two types of predictive mechanistic models: genome-scale metabolic models that have been later applied in **Chapter 2** and **Chapter 3**, and detailed kinetic models, an example of which can be seen in **Chapter 4**. Genome-scale metabolic models (GEMs) represent a whole set of stoichiometry-based, mass-balanced metabolic reactions of an organism (**Chapter 2**) or a specific tissue (**Chapter 3**). Ideally, they consist of all reactions present in the studied organism, tissue, or cell. In reality, auxiliary pathways or metabolism of xenobiotics are often still enigmatic and not studied in enough detail. For example, the metabolism of phytanic acid had not been reconstructed fully in the generic Recon3D model [32]

[1] Arthur Conan Doyle, The Adventure of the Copper Beeches

(**Chapter 3**). This led us to a manual curation and extension of the existing model to represent the most recent knowledge about the phytanic acid breakdown in the cell. The extended model was used to integrate the data obtained from transcriptomics, proteomics, and metabolomics experiments in fibroblasts from healthy volunteers and Refsum disease patients. This fibroblast-specific model represented the interplay between peroxisomes, endoplasmic reticulum, and mitochondria required for the breakdown of phytanic acid. For other applications, however, further curation efforts are required to represent the metabolic complexity of human cells adequately. As introduced in **Chapter 1**, the metabolism of biopolymers remains currently misrepresented in the context of GEMs due to the challenge of a combinatorial explosion created by enzymes promiscuity. Lipidomics studies have shown [33–35] that lipids and phospholipids play a key role in many diseases. Therefore, the solution to the combinatorial problem, which also pertains to lipid metabolism, and an accurate representation of phospholipids should be a top priority.

Furthermore, classical biochemical representation of reactions, represented by simple molecules, can be misleading, as has been shown in **Chapter 2**. In classical biochemistry books, the reaction catalysed by a well-known flavoprotein succinate dehydrogenase is written as

$$succinate + FAD \overset{SDH}{\longleftrightarrow} fumarate + FADH_2 \qquad \text{eq. 5.1}$$

Following this representation, GEMs typically treat flavins (FAD and FMN) and other enzyme-bound cofactors as free-floating metabolites. This allows enzymes to inadvertently oxidise or reduce flavin molecules that are, in reality, confined to another enzyme. In the models, this leads to artificial uncoupling of pathways. In **Chapter 2** I proposed an extensive curation of the Recon 2.2 [36] and Recon 3D models [32] replacing the FAD molecules with the final electron acceptor specific for the reaction, as well as an approach to couple flavoprotein-dependent reactions with flavin synthesis [37]. Taking into the account that ubiquinone (Q) is the final electron acceptor for the succinate dehydrogenase, eq. 5.1 then takes the form of:

$$succinate + Q \overset{SDH:FAD}{\longleftrightarrow} fumarate + QH_2 \qquad \text{eq. 5.2}$$

With the curated model, I was able to accurately simulate the functional consequences of multiple acyl-CoA dehydrogenase deficiency (MADD) and systemic depletion of riboflavin. Moreover, the new model enabled me to predict a larger number of biomarkers in flavoproteome-related diseases, without loss of accuracy. Yet, the true positive rate of biomarker discovery with existing methods remains low. The discrepancy between the accuracy and the true

positive rate can be partially explained by the lack of necessary validation data. To properly validate predictions, we should know not only the truly positive values (the currently known biomarkers) but also the truly negative values (the metabolites known to not significantly change in a particular disease). This step would require scientists to share their negative results more openly to populate the yet non-existing 'True Negative Database'. Once we acquire this information, it will be easier to assess the accuracy of model predictions and to propose truly novel biomarkers for testing. In general, such a database would have the potential to speed up progress in research, as certain ideas will no longer be tested over and over again by many scientists across the globe.

In contrast to the previously used genome-scale modelling approaches, in **Chapter 4** I used a detailed, kinetic model to represent the large neutral amino acids (LNAA) transport via the blood-brain barrier (BBB) and the subsequent neurotransmitter metabolism in the brain. This model was used to test an alternative dietary treatment based on the LNAA supplementation in phenylketonuria (PKU). Metabolic Control Analysis [38–40] of the model has revealed the key players in the pathophysiological mechanism and the impact of individual amino acids in the diet on the underlying brain dysfunction in PKU. However, model validation has also revealed specific discrepancies between model predictions and experimental data. In general, the model was overestimating the impact of high phenylalanine levels, compared to what has been measured in mice. Furthermore, the model showed a smaller effect on the reduction of brain phenylalanine in the diet enriched with leucine and isoleucine than in the experimental data. This inaccuracy provides a valuable insight into the mechanism and rejects the original hypothesis that the observed phenomenon is caused by the high LAT1 affinities for isoleucine and leucine. Rather, it highlights that there might be a different, currently unknown mechanism involved in the action of isoleucine and leucine. A follow-up study focusing specifically on the blood-brain transport of leucine and isoleucine could provide the missing information.

Chapter 4 very clearly illustrated the importance of accurate data in model generation in two major aspects: 1) the lack of species-specific parameters, 2) inadequate validation data. Firstly, the lack of accurate mouse-specific kinetic parameters for the key enzymes could be the culprit of model inaccuracy. Even though it has been more than 100 years since Menten and Michaelis published their paper on the principles of enzyme kinetics [6], our knowledge about the kinetic parameters for many enzymes is incomplete. Furthermore, even if parameters for a given enzyme are available, they have often been measured under non-physiological conditions. However, as discussed by van Eunen and Bakker [41] realistic kinetic models are built to represent

the studied system *in vivo*. Therefore, they should be parametrised using physiological, *in vivo*-like kinetic data specific for the studied organism. Secondly, I used previously published data [42–44] for the validation of the model and designed a model to represent the level of the experimental data complexity. In this first approach to modelling the effect of diet on brain amino acids and neurotransmitter concentrations, it became clear that the experimental data was missing some important information. For example, the experimental dataset consisted of only blood, and total brain concentrations, while the model included blood, endothelial cells (BBB), and brain concentrations. However, since the substrate competition for LAT1 transport across the BBB was hypothesised to be the key mechanism through which proposed diets affected the brain amino-acid levels, the distinction between the endothelial cells and brain compartments had to be made in the model. Therefore, to relate the model outcome to the validation dataset, a weighted average between the blood-brain barrier and the brain compartment was made and an estimate of the cytosolic volume relative to the brain wet weight. Possibly, the uncertainty in this conversion is one of the reasons for the quantitative discrepancy between the model predictions and the experimental data. Furthermore, plasma levels of amino acids (used as an input for model simulations) were reported in different units than the brain amino acid and neurotransmitter concentrations (μmol/L and μmol/g wet weight respectively). Since the conversion between different units used in the experimental study was not possible, the model predictions could only be validated at the level of relative quantitation. To gather the relevant missing information, a new experiment would need to be designed specifically addressing the issues identified by the model. This iterative cycle of experiments and modelling is often seen in systems biology projects [45,46].

To address some of the current experimental shortcomings, a recently developed organ-on-chip technology could be used. It allows studying the interaction between the blood, blood-brain-barrier (BBB), and neurons in a more controlled and uniform manner [47,48]. It gives a unique opportunity to study transport dynamics across not only murine but also human endothelial cells forming the BBB and the effect of it on neuronal cells. If this system would be used, further compartmentalisation of the model to blood (BL), endothelial cells (BBB), cerebral spinal fluid (CSF), and dopaminergic (DA), and serotonergic (HT) neurons could be considered to represent the complexity of the system better. However, organ-on-chip technology may not be suitable to study enzyme kinetics due to the low volumes and cell numbers. Therefore, dedicated enzyme kinetics studies would still need to be performed with isolated murine enzymes in *in vivo*-like media. Furthermore, studies in an isolated and controlled system of organ-on-

chip may be helpful to validate and parametrise the initial model. However, they might not accurately represent the *in vivo* response seen in mice. Consequently, to use the improved model as a tool to design optimal diets, it would have to be validated using *in vivo* data.

As seen in **Chapter 4**, the transport system, even though crucial for communication between compartments, cells, and tissues, requires further studies. The lack of detailed knowledge about transporters has an impact not only on detailed kinetic models but also on the genome-scale models described in **Chapter 2** and **Chapter 3**. Even though kinetic information is not included in the GEMs, the mechanism of transport is important to accurately represent the energy (active transport) and metabolic (facilitated transport, cotransporters, and antiporters) constraints on the network, as well as to connect different compartments of the cell. Currently, for many compounds, a simple diffusion through the lipid bilayer is assumed as a primary transport mechanism. However, new studies show that this assumption may not always be valid, and there might be many unknown transporters involved [49,50].

"We are here, and it is now. The way I see it is, after that, everything tends towards guesswork." [2]

Improved diagnostics and therapeutic approaches tailored to the individual patient are the goals of systems medicine. However, to reach these goals, robust, thoroughly validated generic models are needed that will act as a healthy control to benchmark against in patient-specific analysis. To construct these models, quantitative data comprising of multilevel biochemical network composition and interactions, and various omics describing the components of the network is needed.

Currently, there are two generic human metabolic reconstructions in use: ReconX series [32], and HMR series [51]. Both have been used to study the mechanisms underlying various diseases with some success [52]. However, GEMs generally do not include any regulatory aspects of metabolism, mainly because not all the necessary regulatory or signalling components are known. Furthermore, metabolic regulation plays an important role in the dynamic maintenance of the homeostasis, while GEMs operate under the steady-state assumption. Currently, integration of regulatory networks and dynamic behaviour of the network at a genome-scale has only been addressed in the model of *Mycoplasma genitalium*, one of the smallest bacteria known so far [53]. In their work, Karr et al. constructed 28 submodules of different types (ODEs, flux-

[2] Terry Pratchett, Small Gods

balance analysis, Poisson processes), and connected them by 16 nodes. They assumed that each submodule is independent on a short time-scale (< 1s), therefore can be run independently at each iteration, but would be dependent on the state of the 16 nodes from the previous simulation round [53]. For a human cell, such reconstruction would undoubtedly reach an enormous size, would require many parameters to be experimentally measured, and would depend heavily on intensive computational power and faster algorithms. One of the approaches could consist of integrating currently available detailed kinetic models, like the one created in **Chapter 4** with the GEMs network (**Chapter 2 and 3**) in a quasi-dynamic feedback system similar to the one used by Karr et al.[53]. In such a system, the GEMs network would receive constraints from the detailed model, then calculate a new steady-state and feed the information back to the dynamic model. This approach could allow reducing computational costs while providing a metabolic context for the detailed kinetic model.

Currently used GEMs focus mostly on a single cell or tissue. However, to be able to represent a relationship between the genome and phenotype of a person, we need to invest in building, curating, and validating whole-body models. The first human, gender-specific whole-body models has been recently built by Thiele et al. [54]. It can be parametrised using patient-specific physiological, dietary, and omics data, and allows studies on the interaction between the gut microbiota and their host. An alternative approach has been presented by Krauss et al. [55] who applied dynamic flux-balance analysis to describe human metabolic networks within the context of whole-body physiology-based pharmacokinetic models creating a hybrid model. However, solving a hybrid model is computationally more expensive (due to iteration) than solving a single linear or quadratic problem in whole-body models. If the biological question does not require the dynamical consideration, then the stoichiometric whole-body models will be a valuable alternative.

All currently used models have been proven useful in providing insights into human metabolism. Since they try to uncover the basic, general principles behind the manifested high-level phenotype, they could be used across many diseases and populations. Even the detailed kinetic models, such as the model of transport of amino acids across the blood-brain barrier discussed in **Chapter 4**, can be used in studies of many different diseases where amino acid and neurotransmitter metabolism is affected, such as Parkinson's disease [56] or Alzheimer disease [57]. However, to be used in the clinic, they have to be rigorously validated using the FDA procedures [58]. This would require more studies being performed where person-specific data would

be integrated with either a single-cell or a whole-body model, and the results would be benchmarked against the "healthy" phenotype. These studies would require access to a big amount of omics data to achieve first the "healthy" model. Then careful, dedicated validation experiments testing both true positive and true negative differences would have to be performed. Fortunately, with the current developments in omics data and computational technologies, this step can be applied in the near future.

In conclusion, the work presented in this thesis demonstrates that applying a systems medicine approach to studies of inborn errors of metabolism could provide not only valuable insights in the mechanism of the studied diseases but also create robust, generic models applicable to other studies of human metabolism. In the future, these generic models, aided by the integrative network analysis and multi-omics data, may provide invaluable insight into the complex metabolic networks and provide a platform to introduce patient-specific data-driven diagnosis and therapies.

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